

The Effect of the Soybean Trypsin Inhibitor on the Enzymatic Release of Amino Acids from Autoclaved Soybean Meal ¹

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INTRODUCTION

It has been well established that proper heat treatment or methionine supplementation will effect a substantial improvement in the nutritive value of raw soybean (1-3). Melnick *et al.* (4) have suggested that, in the case of raw soybean, methionine is liberated in the gastrointestinal tract too late to effect mutual supplementation of the other essential amino acids. Considerable attention has been directed to the presence of one or more trypsin inhibitors in raw legumes (5-10), and the deleterious effect of such inhibitor concentrates on the growth of experimental animals (11-16). The foregoing evidence would strongly suggest that the poor growth-promoting qualities of raw soybean may be attributed to an inhibition of *in vivo* enzymatic digestion by the antitryptic factor, which, according to the hypothesis of Melnick *et al.*, must specifically retard the release of methionine.

¹ This investigation, undertaken by one of us (I. E. L.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Southern California, reports research conducted at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 224 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

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The present paper reports the results of an investigation involving a study of the effect of the soybean trypsin inhibitor on the enzymatic⁴ release of amino acids from autoclaved soybean meal. Raw and autoclaved soybean meal without added inhibitor provided a suitable basis for comparison. A comparison of the effect of the trypsin inhibitor on the release of amino acids from heat-processed and from raw soybean meal is valid only if a suitable quantity of the inhibitor is added to the properly heated soybean meal to reduce its digestibility to that of the meal before heating. The initial phase of this study, therefore, deals with the determination of the level of the inhibitor necessary for its replacement in heated soybean meal to obtain a rate of digestibility substantially equivalent to that prior to heating. The second phase is concerned with the effect of this level of the inhibitor on the release of several amino acids from autoclaved soybean meal. Finally, the effect of preliminary peptic digestion on the ability of the inhibitor to retard the digestion and release of methionine from autoclaved soybean meal was determined.

EXPERIMENTAL

The soybean used was of the Bansei variety. The beans were finely cominuted and extracted with chloroform at room temperature, which reduced its fat content from 24% to 2%. The resulting meal, autoclaved at 15 lb. pressure (115°C.) for 20 min., was found to exhibit maximum digestibility and a negative test for the antitryptic factor as determined by the method of Westfall and Hauge (13). This degree of autoclaving was a satisfactory compromise among the several time-temperature relationships reported to produce maximum nutritive value (13, 17-20).

The inhibitor extract was prepared in the following manner. A 20% suspension of the unheated meal was adjusted to pH 4.2 with concentrated HCl and stirred overnight in the cold with an electric stirrer. The suspension was filtered through cheese cloth and subsequently through rapid filter paper. The filtrate was centrifuged to remove any insoluble material that precipitated upon standing in the cold. The supernatant, designated as the inhibitor extract, contained approximately 1.5 mg. N/ml. The extract was preserved under toluene in the refrigerator, and no loss in activity was observed throughout the period of study.

The Effect of the Trypsin Inhibitor on the Digestibility of Autoclaved Soybean Meal

The *in vitro* digestion technique employed was essentially that proposed by Melnick *et al.* (4, 21). The procedure, in brief, was as follows. Samples of autoclaved soybean meal weighing 13 g. (equivalent to 6 g. of protein) were accurately weighed and transferred

⁴ The term "enzymatic," as used here, refers to the proteolytic activity of pancreatin (U. S. P. purchased from Central Scientific Company, Chicago, Illinois).

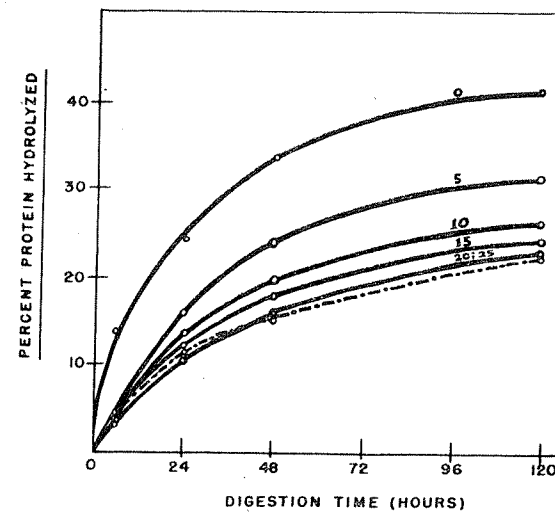


FIG. 1. The effect of increasing increments of soybean inhibitor extract on the *in vitro* digestibility of autoclaved soybean compared to raw soybean. Autoclaved soybean is represented by the smooth curve, the accompanying numerals indicating the ml. of inhibitor extract added. Raw soybean is represented by the dot-dash curve.

to 100 ml. glass-stoppered graduate cylinders. Approximately 75 ml. of borate buffer, pH 8.3, were added to each cylinder and the mixture thoroughly shaken. After being diluted to the mark with the buffer, the mixture was transferred to a 250 ml. glass-stoppered bottle. An aliquot (5-25 ml.) of the inhibitor extract diluted to 25 ml. with distilled water was added to the digestion mixture. The addition of 10 ml. of 2% pancreatin and 15 ml. of buffer brought the final volume to 150 ml. Ten ml. of toluene were added to minimize bacterial growth during subsequent incubation at 37°C. Similar mixtures of raw and autoclaved soybean meal to which inhibitor had not been added and enzyme controls containing various levels of inhibitor were run concurrently. Formol titrations were conducted on 5 ml. aliquots removed at intervals of 0, 5, 24, 48, and 120 hr. The per cent protein hydrolyzed⁵ is compiled in Table I and graphically presented in Fig. 1.

⁵ Calculated by using the formula:

$$\frac{a - b}{c - b} \times 100,$$

where c = formol titration of 5 ml. of acid hydrolyzate obtained by refluxing 13 g. of soybean with 75 ml. of 8 N H₂SO₄ for 24 hr. and subsequently diluted to 150 ml.

b = formol titration at time $t = 0$.

c = formol titration at time t .

TABLE I

The Effect of Soybean Inhibitor Extract on the Digestibility of Autoclaved Soybean

Soybean treatment	Inhibitor extract	Digestion period	Protein hydrolysis	Inhibition	$i/I^a \times 10^2$
None (raw)	0	hr. 5	per cent 2.1	per cent 84.0	
		24	11.6	51.7	
		48	16.1	52.1	
		120	22.7	46.0	
Autoclaved	0	5	13.2	0	
		24	24.0	0	
		48	33.7	0	
		120	42.1	0	
	5	5	4.0	69.6	7.2
		24	16.2	32.5	15.4
		48	24.1	28.3	17.6
		120	31.2	25.9	19.3
	10	5	2.6	80.4	12.4
		24	13.3	44.5	22.5
		48	20.0	40.6	24.6
		120	26.6	36.8	27.1
	15	5	2.9	78.7	19.1
		24	12.4	48.5	31.0
		48	18.3	45.5	33.0
		120	24.1	42.7	35.1
	20	5	2.4	81.8	24.5
		24	10.7	55.5	36.1
		48	16.8	50.2	39.8
		120	22.9	45.5	44.0
	25	5	1.8	86.2	29.0
		24	11.0	54.0	46.3
		48	17.1	49.3	50.7
		120	22.9	45.5	55.0

^aMl. inhibitor extract/% inhibition.

It will be noted from Fig. 1 that increasing increments of the inhibitor extract produced a decreasing rate of digestibility of the autoclaved soybean meal, and that 20–25 ml. of the inhibitor solution were sufficient to reduce its digestibility to approximately the same level as that of the unheated meal.

The experimental data, moreover, provided a means of verifying from a theoretical basis the level of the inhibitor extract necessary to add to the autoclaved soybean meal to obtain a digestibility equivalent to that before heating. Applying the fundamental concepts of Michaelis and Menten (22), that substrate and enzyme form an

intermediate complex, and of Northrop (23), that the inhibitor competes with the substrate by combining with the enzyme in accordance with the law of mass action, an expression can be derived (24) relating the per cent inhibition of digestion (per cent decrease in the digestion of autoclaved soybean meal due to the presence of the inhibitor) as a function of the level of the inhibitor extract. This relationship can be expressed by the equation:

$$I = \frac{i}{c + ki} \quad (1)$$

where i = ml. of inhibitor extract

$$I = \text{per cent inhibition} = \frac{A - B}{A} \times 100$$

A = per cent of the autoclaved soybean meal protein hydrolyzed;

B = per cent of the autoclaved soybean meal protein hydrolyzed when i ml. of inhibitor extract are added; or of raw soybean meal protein

c and k = empirical constants.

If the reciprocal of Eq. (1) is multiplied by i we obtain the expression:

$$i/I = c + ki. \quad (2)$$

Eq. (2) represents a straight line when i/I is plotted against i , the intercept on the i/I axis and the slope determining the constants c and k , respectively.

In order to evaluate the experimental data in terms of theoretical predictions, the values for i/I are included in Table I. In Fig. 2, i/I vs. i is plotted for the digestion periods of 24, 48, and 120 hr. Such data appeared to exhibit rather closely the linear relationship predicted by Eq. (2). The constants c and k , expressed as the intercept on the i/I axis and the slope, respectively, are recorded in Table II. Since the percent inhibition, I , exhibited by the unheated meal, is known for each digestion period (see Table I), it is now possible to calculate from Eq. (1) the corresponding concentration of the inhibitor extract, i , necessary to effect this degree of inhibition. The results of such calculations in Table II give an average value for i of 20.3, which is within the range of 20 to 25 ml. of inhibitor extract observed in Fig. 1 as being necessary to add to autoclaved soybean meal to produce the same rate of digestibility as that of the unheated protein.

The values for constants c and k were inserted into Eq. (1) in order to calculate the theoretical curves shown in Fig. 3, where percent inhibition, I , is plotted against ml. of inhibitor solution, i . Experimental data taken from Table I are likewise plotted for comparison.

The Effect of the Trypsin Inhibitor on the Release of Amino Acids from Autoclaved Soybean Meal

The rate of release of several amino acids was studied on raw and autoclaved soybean meal, and on the latter to which 25 ml. of inhibitor extract⁶ had been added. The previously described digestion technique was used with the modification that, in

⁶ For the purpose of convenience it was decided to use 25 ml. of inhibitor extract, this being justified by the fact that the difference in digestibility effected by 20 or 25 ml. was negligible (see Figs. 1 and 3).

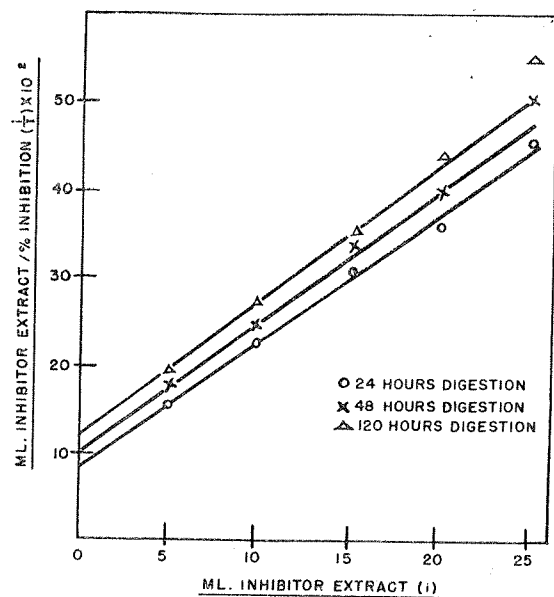


FIG. 2. Graphic determination of the constants c and k in the equation: $i/I = c + ki$ where c is the intercept on the i/I axis and k is the slope of the curve. (See text for details.)

TABLE II

Concentration of Inhibitor Extract Necessary to Produce a Degree of Digestibility in Autoclaved Soybean Equivalent to Raw Soybean

Digestion period	$c \times 10^2$ ^a	$k \times 10^2$ ^b	Inhibition of raw soybean ^c	i , inhibitor extract ^d
24 hr.	8.5	1.47	per cent	ml.
48 hr.	10.0	1.50	51.7	18.0
120 hr.	11.5	1.57	52.1	23.6
Average	10.0	1.51	46.0	19.2
			49.9	20.3

^a Intercept on i/I axis, Fig. 2.

^b Slope of curve in Fig. 2.

^c Taken from Table I.

^d Calculated from Eq. (1) in text.

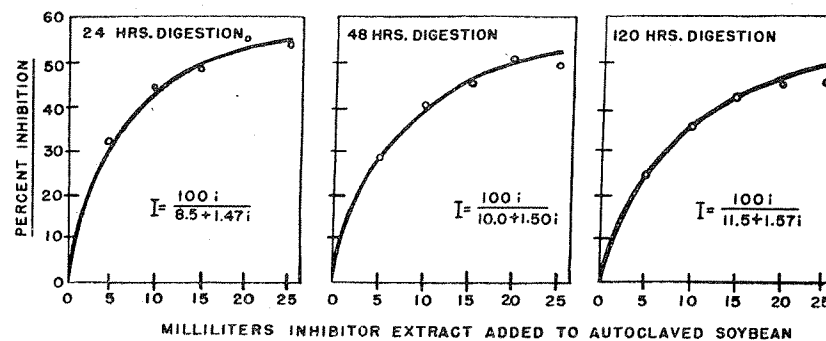


FIG. 3. A comparison of experimental data and theoretical equations based on assumption that enzyme and inhibitor form a reversible complex in accordance with the law of mass action. Curve is theoretical based on equation shown in each graph; circles represent experimental data taken from Table I.

addition to removing aliquots for formol titration at stated intervals of time, aliquots of the digestion mixture removed at the same time were placed in a steam sterilizer for 15 min. to inactivate the proteolytic enzymes. The undigested protein was precipitated by adjusting the pH to 4.2 with several drops of glacial acetic acid. The clear filtrates were preserved in the cold with toluene and subsequently used for amino acid analyses. For the latter, the microbiological techniques indicated below were so modified that the semimicro procedure of Sauberlich and Baumann (25) was applicable in all cases. *Leuconostoc mesenteroides* was used to determine aspartic acid (25), lysine (26), and methionine (27); *Lactobacillus arabinosus* was employed for the assay of isoleucine (28), leucine (29), tryptophan (30), and valine (29). The per cent amino acid released was computed by dividing the amount of the amino acid released by enzymatic hydrolysis at time t , by the total amino acid released by acid (in the case of tryptophan, "alkaline") hydrolysis from raw or autoclaved soybean meal and multiplying by 100. The results of these assays are presented in graphs in Fig. 4.

Effect of Preliminary Peptic Digestion

The susceptibility of the crystalline soybean inhibitor to peptic inactivation has been reported by Kunitz (10). *In vitro* digestion experiments by Evans and his co-workers (31,32) showed that the proteins of raw and autoclaved soybean were digested and the methionine released to approximately the same extent when tryptic digestion was preceded by digestion with pepsin. It was deemed advisable, therefore, to determine directly the effect of preliminary peptic digestion on the enzymatic release of methionine from autoclaved soybean meal to which the inhibitor had been added, the heated and unheated meals without added inhibitor again providing a suitable basis for comparison.

The digestion procedure remained the same except that 0.1 N HCl and 0.1 g. of pepsin (1:10,000) were substituted for the buffer and pancreatin, respectively. After

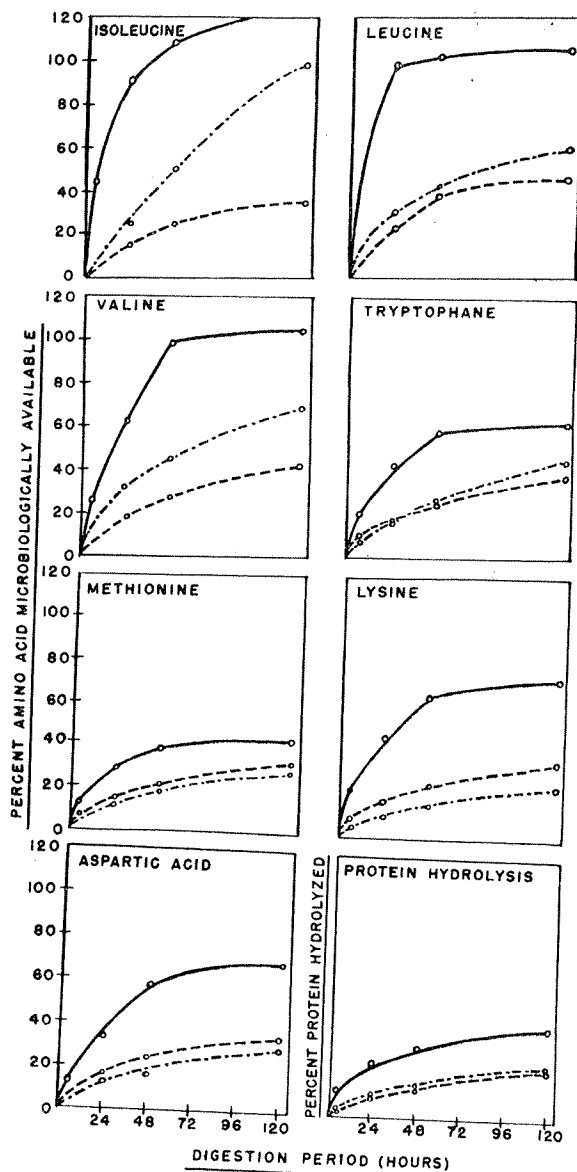


FIG. 4. Rate of release of microbiologically available amino acids from autoclaved soybean (smooth curve), raw soybean (broken curve), and autoclaved soybean plus inhibitor (dot and dash curve). Digestibility is shown for comparison.

48 hr. incubation at 37°C., the mixture was adjusted to a pH of 8-9 with a few drops of saturated NaOH. After the addition of 0.2 g. of pancreatin, followed by a further incubation period of 96 hr., formol titrations and microbiological assays for methionine were conducted. These results are tabulated in Table III.

TABLE III
The Effect of Preliminary Peptic Digestion on the Digestibility and Release of Methionine from Soybean

Method of digestion	Raw	Autoclaved	Autoclaved +inhibitor ^b
Per cent protein digested			
Pancreatin ^a	23.6	43.0	24.2
Pepsin and pancreatin	42.8	43.0	35.7
Per cent methionine released			
Pancreatin ^a	33.0	42.0	29.2
Pepsin and pancreatin	70.5	64.0	60.5

^a Preliminary digestion with heat inactivated pepsin.

^b 25 ml. of inhibitor extract.

DISCUSSION

From Fig. 3 it is evident that the experimental data are in good agreement with the theoretical assumption that the mechanism of the inhibition of the digestion of soy protein by pancreatin consists in the formation of an enzyme-inhibitor complex reversibly dissociating in accordance with the law of mass action. These results, as well as those reported by Borchers *et al.* (33), are at variance with the observation by Kunitz (10), that crystalline soybean inhibitor and crystalline trypsin form an irreversible stoichiometric compound. The conditions of the experiments reported here are not quite comparable in all respects since the enzyme preparation used, pancreatin, may contain a variety of proteolytic enzymes, and the crude inhibitor may include a number of antitryptic factors (7,8) which may or may not combine with these proteolytic enzymes in the manner described by Kunitz for his crystalline materials.

In confirmation of previously reported results (4,34), Fig. 2 shows that the release of all the amino acids studied proceeded at a slower rate from the unheated meal than from the autoclaved material. There was no evidence, however, to indicate that methionine was specifically retarded in its release from the unheated soybean meal. Riesen *et al.*

(34) have also stated that . . . "heat treatment increased the rate of liberation of methionine, however, not proportionately more than it increased the rate of liberation of the other essential amino acids, excluding lysine."

Although the effect of the inhibitor extract on autoclaved soybean meal was to produce an overall rate of digestibility substantially equivalent to the raw meal (see "per cent protein hydrolyzed"—Fig. 4), the release of the individual amino acids varied above and below the level of release of the corresponding amino acids of the raw meal. If the curves of the autoclaved protein to which the inhibitor had been added are compared with the curves of the raw soybean protein in Fig. 4, it can be noted that the rates of release of lysine, in particular, and methionine and aspartic acid, to a lesser degree, are less than the corresponding rates before heating. On the other hand, the rates of release of the other amino acids, isoleucine and valine especially, are greater.

The explanation for these differences undoubtedly lies in the fact that the effect of heat on soybean is two-fold: (1) inactivation of the trypsin inhibitor, and (2) alteration of the physical properties of the soybean protein *per se* (35), which is quite independent of the inhibitor. Since, under the conditions of this experiment, the trypsin inhibitor which had been inactivated by heat is in effect replaced, any differences in the rate of release of amino acids from raw and autoclaved soybean meal to which the inhibitor had been added may be attributed to the effect of heat on the protein. In this connection, Riesen *et al.* (34) have similarly noted that soybean meal autoclaved for 4 min. released all of its essential amino acids except lysine at a faster rate than the raw meal to which sufficient pancreatin had been added to overcome the effect of the trypsin inhibitor. Under the conditions reported here, that is, autoclaving soybean meal for 20 min., methionine and aspartic acid as well as lysine become more refractory to enzymatic release; nevertheless, this same treatment still facilitated the release of leucine, isoleucine, valine, and possibly tryptophan, to a greater degree than can be explained by inactivation of the inhibitor alone. Since the release of all amino acids is adversely affected by excessive heat (34), it becomes apparent that there must exist for each amino acid a critical degree of exposure to heat, below which its release is facilitated and above which its release is retarded; whichever effect occurs, however, is usually obscured by the concomitant inactivation of the trypsin inhibitor.

It is interesting to note that the two amino acids which were observed

to be most vulnerable to heat, lysine and methionine, also tend to become the first limiting amino acids causing the lowered nutritive value of overheated soybean meal (17-19).

Table III indicates that preliminary digestion with pepsin obscures the differences in digestibility and the release of methionine between raw and autoclaved soybean meal, an effect which may be ascribed primarily to the action of pepsin on the inhibitor.

Evidence has been presented in recent papers (14,15) to show that trypsin inhibitor concentrates exert their major growth-inhibiting effect through a mechanism not directly related to an inhibition of normal enzymatic hydrolysis in the intestinal tract. Such an observation might be expected if the trypsin inhibitor were destroyed or inactivated by peptic digestion in the stomach of the rat. It is difficult to determine to what extent our *in vitro* results can be extended to conditions *in vivo*. It may be significant to note, however, that most experiments with rats thus far reported have yielded results comparable to those obtained by serial enzymatic digestion shown in Table III, that is, the slight difference in digestibility (4,36) as well as the ultimate intestinal absorption of methionine (4,37) between raw and autoclaved soybean.⁷ It is possible, therefore, that, because of peptic inactivation in the stomach, the trypsin inhibitor may be nonoperative *in vivo* and the poor growth-promoting quality of raw soybean may be attributed, at least in part, to a heat-labile substance closely associated with it. Animal experimentation is now in progress to determine the validity of this hypothesis.

SUMMARY

1. Experimental and theoretical data provide a means for determining the amount of soybean trypsin inhibitor extract to add to autoclaved soybean meal to produce a degree of digestibility equivalent to that of the unheated meal.

2. The inhibition of the digestion of soybean protein appears to involve enzyme and inhibitor in reversible equilibrium in accordance with the law of mass action.

⁷ That the trypsin inhibitor is still active in the intestinal tract of the chick was shown by Ham *et al.* (11). In contrast to rats, chicks exhibit marked differences in digestibility and absorption of methionine between raw and autoclaved soybean (32,38). It was suggested by Evans (32) that this species difference may be due to poor gastric digestion in the chick, which approximates *in vitro* digestion by trypsin and erepsin, and the higher degree of peptic digestion in the rat.

3. The release of methionine from raw soybean meal was not found to be specifically retarded by the trypsin inhibitor but followed the same general pattern of inhibition evidenced by other amino acids.

4. The antitryptic factor does not fully account for the difference in the release of amino acids from raw and autoclaved soybean meal. In addition to the inactivation of the antitryptic factor, alteration in the soybean protein as a result of heat may retard or facilitate the release of amino acids.

5. Peptic inactivation of the trypsin inhibitor masks differences in digestibility and release of methionine between raw and autoclaved soybean meal. The possible nutritional significance of these results is discussed.

REFERENCES

1. BARNES, R. H., AND MAACK, J. E., Review of the Literature on the Nutritive Value of Soybeans, The Hormel Inst. of the Univ. of Minnesota, May, 1944.
2. HAYWARD, J. W., AND HAFNER, F. H., *Poultry Sci.* **20**, 139 (1941).
3. ALMQUIST, H. J., MECCHI, E., KRATZER, F. H., AND GRAU, C. R., *J. Nutrition* **24**, 385 (1942).
4. MELNICK, D., OSER, B. L., AND WEISS, S., *Science* **103**, 326 (1946).
5. HAM, W. E., AND SANDSTEDT, R. M., *J. Biol. Chem.* **154**, 505 (1944).
6. BOWMAN, D. E., *Proc. Soc. Exptl. Med.* **57**, 139 (1944).
7. BOWMAN, D. E., *ibid.* **63**, 547 (1946).
8. BOWMAN, D. E., *Arch. Biochem.* **16**, 109 (1948).
9. KUNITZ, M., *J. Gen. Physiol.* **29**, 149 (1946).
10. KUNITZ, M., *ibid.* **30**, 291 (1947).
11. HAM, W. E., SANDSTEDT, R. M., AND MUSSEHL, F. E., *J. Biol. Chem.* **161**, 635 (1945).
12. KLOSE, A. A., HILL, B., AND FEVOLD, H. L., *Proc. Soc. Exptl. Biol. Med.* **62**, 10 (1946).
13. WESTFALL, R. J., AND HAUGE, S. M., *J. Nutrition* **35**, 379 (1948).
14. KLOSE, A. A., GREAVES, J. D., AND FEVOLD, H. L., *Science* **108**, 88 (1948).
15. WESTFALL, R. J., BOSSHARDT, D. K., AND BARNES, R. H., *Proc. Soc. Exptl. Biol. Med.* **68**, 498 (1948).
16. BORCHERS, R., ACKERSON, C. W., AND MUSSEHL, F. E., *Arch. Biochem.* **19**, 317 (1948).
17. CLANDININ, D. R., CRAVENS, W. W., ELVEHJEM, C. A., AND HALPIN, J. G., *Poultry Sci.* **26**, 150 (1947).
18. FRITZ, J. C., KRAMKE, E. H., AND REED, C. A., *ibid.* **26**, 657 (1947).
19. KLOSE, A. A., HILL, B., AND FEVOLD, H. L., *Food Technol.* **2**, 201 (1948).
20. BORCHERS, R., ACKERSON, C. W., AND MUSSEHL, F. E., *Poultry Sci.* **27**, 601 (1948).
21. MELNICK, D., AND OSER, B. L., *Food Technol.* in press.
22. MICHAELIS, L., AND MENTEN, M. L., *Biochem. Z.* **49**, 333 (1913).
23. NORTHROP, J. H., *J. Gen. Physiol.* **4**, 245 (1922).
24. NORTHROP, J. H., KUNITZ, M., AND HERRIOTT, R. M., Crystalline Enzymes. Columbia Univ. Press, New York, 1948, p. 157-8.
25. SÄUBERLICH, H. E., AND BAUMANN, C. A., *J. Biol. Chem.* **166**, 417 (1946).
26. DUNN, M. S., CAMIEN, M. N., SHANKMAN, S., FRANKL, W., AND ROCKLAND, L. B., *ibid.* **156**, 715 (1944).
27. LYMAN, C. M., MOSELY, O., BUTLER, B., WOOD, S., AND HALE, F., *ibid.* **166**, 161 (1946).
28. KUIKEN, K. A., NORMAN, W. H., LYMAN, C. M., HALE, F., AND BLOTTER, L., *ibid.* **151**, 615 (1943).
29. SCHWEIGERT, B. S., MCINTIRE, J. M., ELVEHJEM, C. A., AND STRONG, F. M., *ibid.* **155**, 183 (1944).
30. GREENE, R. D., AND BLACK, A., *Proc. Soc. Exptl. Biol. Med.* **54**, 322 (1943).
31. EVANS, R. J., *Arch. Biochem.* **11**, 15 (1946).
32. EVANS, R. J., MCGINNIS, J., AND ST. JOHN, J. L., *J. Nutrition* **33**, 661 (1947).
33. BORCHERS, R., ACKERSON, C. W., AND SANDSTEDT, R. M., *Arch. Biochem.* **12**, 367 (1947).
34. RIESEN, W. H., CLANDININ, D. R., ELVEHJEM, C. A., AND CRAVENS, W. W., *J. Biol. Chem.* **167**, 143 (1947).
35. EVANS, R. J., AND ST. JOHN, J. L., *J. Nutrition* **30**, 209 (1945).
36. HAYWARD, J. W., STEENBOCK, H., AND BOHSTEDT, G., *ibid.* **11**, 219 (1936).
37. JOHNSON, L. M., PARSONS, H. T., AND STEENBOCK, H., *ibid.* **18**, 423 (1939).
38. EVANS, R. J., AND MCGINNIS, J., *ibid.* **31**, 449 (1946).